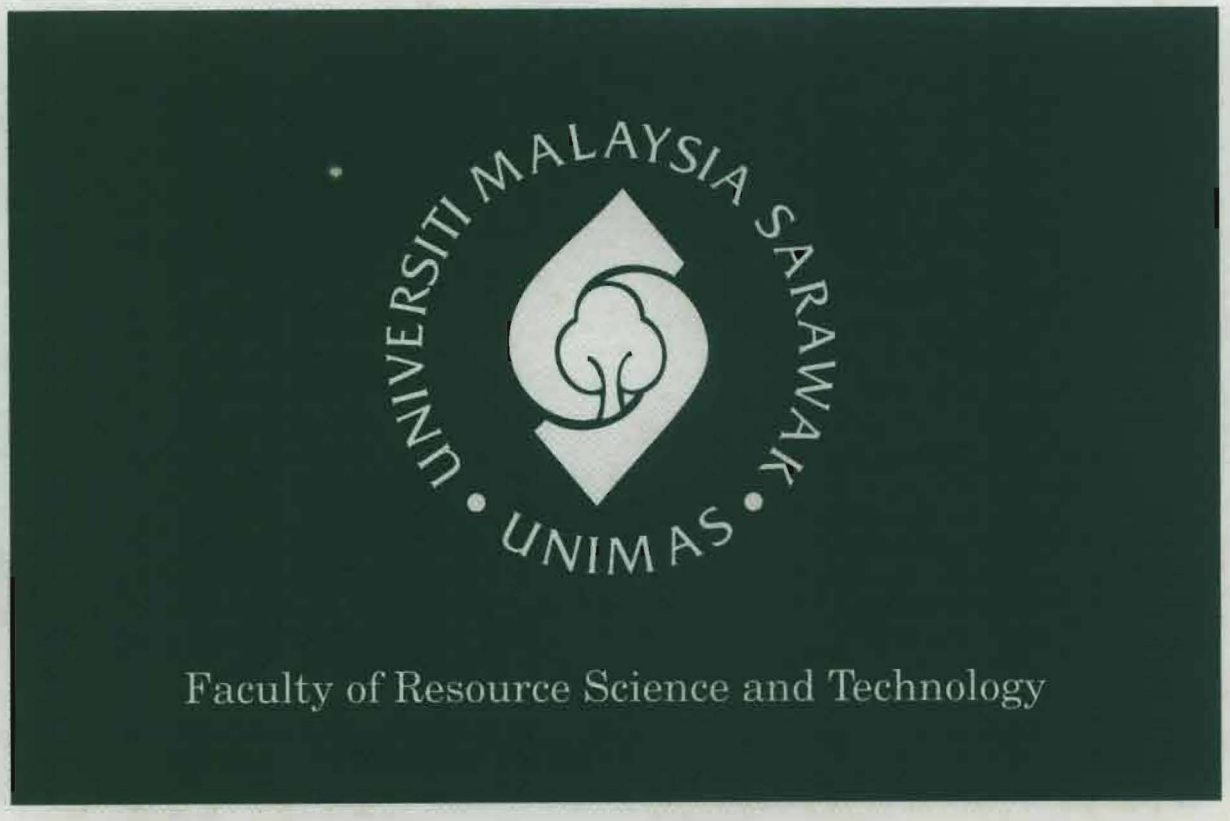

CLONING OF ADP-GLUCOSE PYROPHOSPHORYLASE INVOLVES IN STARCH BIOSYNTHESIS PATHWAY IN SAGO PALM

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Cloning of ADP-glucose Pyrophosphorylase Involves in Starch Biosynthesis Pathway in Sago Palm

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ABSTRACT

The main objective in this study is to clone ADP-glucose pyrophosphorylase, which is a gene that involves in starch biosynthesis pathway. In order to accomplish this objectives, three main steps which are the isolation of sago genomic DNA, Polymerase Chain Reaction and cloning putative ADP-glucose Pyrophosphorylase need to be done. The first two steps had been done but unfortunately the last step could not be proceed due to the failure to obtain any PCR product.

ABSTRAK

Objectif utama dalam kajian ini adalah untuk mengklon ADP-gluco pyrophosphorylase yang merupakan gen yang terlibat dalam laluan biosintesis kanji. Untuk mencapai objektif ini, tiga langkang utama iaitu pemencilan genom DNA sagu, PCR dan pengklonan gen ADP-glucose pyrophosphorylase perlu dilakukan. Langkah pertama dan kedua telah dilakukan tetapi langkah ketiga tidak dapat diteruskan kerana kegagalan untuk mendapat produk PCR pada langkah kedua.

CHAPTER 1 : LITERATURE REVIEW

1.1 Introduction

Sago palm (*Metroxylon sagu*), better known as 'rumbia', is commonly distributed in Southeast Asia region. It has long been recognized as an important natural resource in daily livelihood of rural peoples because of its multiple usage. Sago is potentially a veery important starch producing tree which grows well in the swampy lowlands with minimum care (Othman,1991). Sagu has been planted commercially specially in Papua New Guinei, Indonesia, Malaysia, Thailand and Philipines, where the total acreage for this crop has been estimated to be more than 3.75 million hectares (Flach,1997)

In Malaysia, Sarawak has been the main state for sago plantationwhereby there are about 1.4 million hectares of peat soil area, suitable for plantation of this crop. The total acreage of sago in Sarawak is about 20000 ha and about 75% of the sago planting areas are located in Mukah, Igan and Oya-Dalat districts of Sibu division and Balingian. It has been estimated that Sarawak has been producing about 55,000 tones of sago starch each year (Tie *et al.*,1991)

Sarawak has been the principal exporter of sago starch to the world market. The monopoly is basically due to the well establish sago cultivation areas available and the large scale production of sago starch practiced in this site (Zulpilip *et al.*,1991)

The high commercial value of sago starch has generated interest among a number of countries in the region to carry out studies on the sago palm. In order to improve the production of sago starch, commercial cultivation using modern state management techniques has been initiated by Sarawak's Land Custody and Development Authority (LCDA). A research unit of the LCDA, known as Crop and Research Application Unit (CRAUN) has been set up to conduct research and development on sago planting and commercialization (Kee, 2001).

1.2 Biochemical Pathway For Starch Biosynthesis in Plants

Starch is a mixture of two polymers, amylose, a linear (1-4) – linked – α-D-glucan and amylopectin a highly branched molecule which consists of short chains of (1-4) – linked – α-D-glucose with (1-6)-linked branches. Chain length of amylose are commonly in excess of 6000 D-glucopyranose units with molecular weight between 150 000 and 600 000 Da (Ahmad *et al.*,1991; Kennedy *et al.*,1983)

Starches are found primarily in storage organs such as tubers and seeds. It is synthesized in specialized plastids known as amyloplasts and also in chloroplast. Starch is a major component of average dietary intake of man and animals. As well as its uses in nutrition, starch is also an important component in manufacturing wide range of industrial products such as paper, textiles and building materials. Furthermore, chemically modified starch and starch derivatives are used widely throughout industry. Starch also plays a major role

in metabolisms in most higher plants since it serves as the plant major food reserve.

There are three amyloplast-specific enzymes involved in starch biosynthesis pathway. They are, ADP-Glucose pyrophosphorylase (AGPase), starct synthase and starch branching enzyme. ADP-glucose pyrophosphorylase is a key enzyme in the biosynthesis of starch in plants. AGPase catalyzes the conversion of glucose-1-phosphate to ADP-glucose, the substrate of starch polymers.

Figure 1 shows the starch biosynthesis pathway in non-photosynthetic, starch storing organ.

1.3 ADP- glucose Pyrophosphorylase (AGPase)

ADP-glucose pyrophosphorylase (AGPase, EC 2.7.7.27), is a key enzyme in the biosynthesis of starch in plants and glycogen in bacteria. AGPase catalyzes the conversion of glucose-1-phosphate to ADP-glucose, the substrate of starch polymers (amylose and amylopectin) (Thorbjornsen *et al.*,2000). AGPase in higher plants is a heterotetramer composed of two small and two large subunits. In the study of comparing the primary structures of 11 plant AGPase protein by Smith-White and Preiss (1992}, they suggested the existence of at least three

types of AGP polypeptides ; (1) small subunit that is found in both the photosynthetic and non-photosynthetic tissues ; (2) large subunit that is found exclusively in photosynthetic tissues; and (3) large subunit that is present exclusively in photosynthetic tissues (AU S.L *et al.*,2002). It is known that AGPase expressed both in tubers (non-photosynthetic) and leaves (photosynthetic) (Anderson *et al.*, 1989; Villand *et al.*, 1992 ; villand *et al.*, 1993).

1.4 Objectives

i. To isolate and clone the putative AGPase gene.

ii. To study the structure and function of the AGPase.

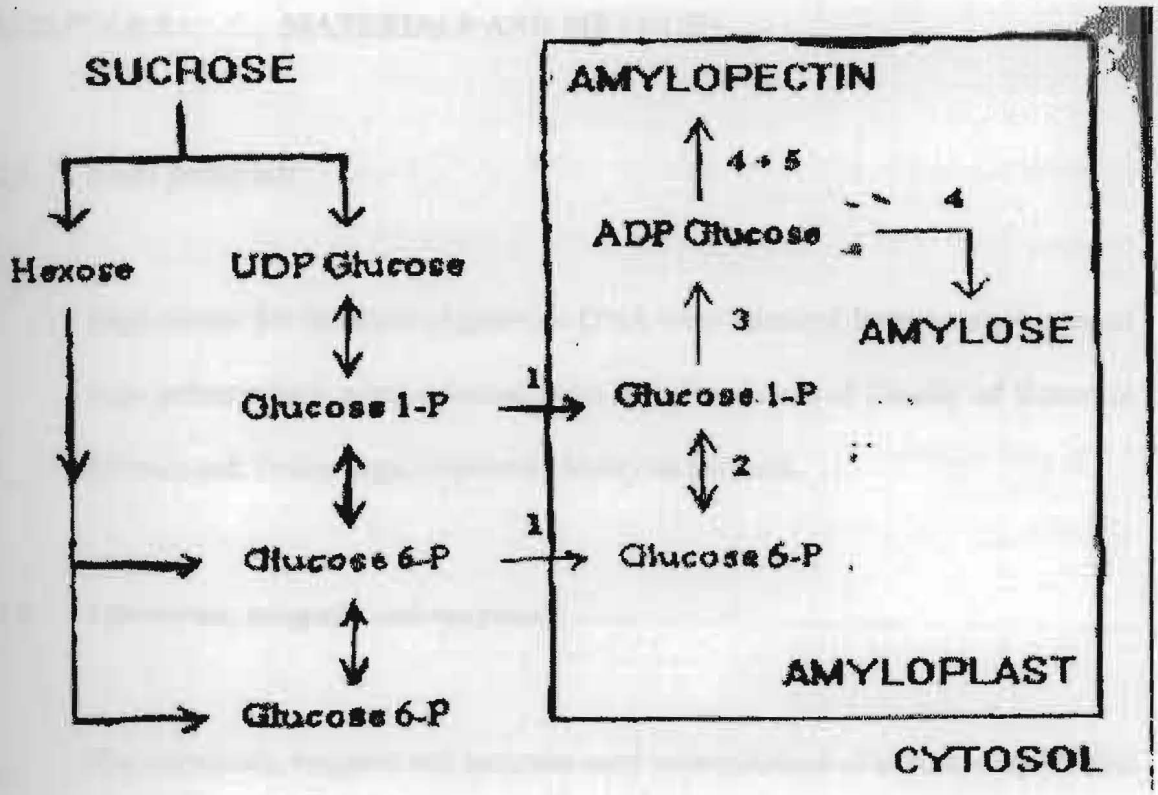

Figure 1. The starch syntehesis pathway in non-photosynthetic, starch storing organs. The enzymes involved are; 1, Hexose –phosphate translocator; 2, plastidial phosphoglucomutase; 3, ADP-Glucose pyrophosphorylase; 4, starch synthase; 5' starch-branching enzyme. The hexose phosphate crosses the amyloplast envelope. Inside the amyloplast, ADP-glucose is synthesized via ADP-glucose pyrophosphorylase. ADP-glucose is the substrae for the synthesis of starch polymers amylose and amylopectin via isoforms of starch synthase and starch branching enzymes. (Taken from Smith *et al.*, 1995)

CHAPTER 2 : MATERIALS AND METHODS

2.1 Plant materials

Sago leaves for isolation of genomic DNA were obtained from young leaves of sago palms which were collected from the plant house of Faculty of Resource Science and Technology, University Malaysia Sarawak.

2.2 Chemicals, reagents and enzymes

The chemicals, reagents and enzymes used were obtained of analytical grade and molecular grade.

2.3 General laboratory procedures

All reuseable laboratory apparatus (glassware, pestles and mortar, spatulas) were washed well with detergent and then sterilized by autoclaving at 121° C for 15 minutes. Pippet tips, microcentrifuge tubes were of Dnase- and Rnase-free disposable type.

2.4 Isolation of genomic DNA

DNA was isolated by using the CTAB Extraction Method (Doyle and Doyle, 1990) with some modification by ACM Gillies. About 2 to 3g of fresh leaf was ground to a powder in liquid nitrogen by using mortar and pestle. The powder was then transferred into preheated 2X CTAB buffer (100mM Tris HCL pH 8.0, 20mM EDTA pH 8.0, 1.4M Nacl, 2% w/v CTAB, 1% w/v PVP with 0.2% β-mercaptoethanol). The mixture were mixed well before being incubated at 60°C for 1 to 2 hours with optional occasional gently swirling.

After incubation, the samples were extracted once with some volume of chloroform : isomylalcohol (24:1) then was centrifuge at 4000 rpm for 15 minutes at room temperature to the concentrate phases. The top phase was carefully transferred to a clean centrifuge tube and 2/3 volume of ice-cold isopropanol was added to precipitate the DNA.

After precipitation, the DNA was palleted by centrifugation at 12000 rpm for 10 minutes. The supernatant was then poured off and the pallet was washed with cold 70% ethanol. Tubes were stored at -20°C for about 30 minutes before centrifuge (13000 rpm, 2 minutes). Then the pallet was resuspended in 50µl of TE buffer.

2.5 Qualitative estimation of DNA by agarose gel electrophoresis

5µl of the DNA preparation was loaded with 2µl of 6X Gel Loading Dye into a 0.8% agarose gel with 2µl of ethidium bromide incorporated. λ *Hind*III DNA ladder was also loaded besided the DNA sample as a marker. The gel electrophoresis was carried out at 70 volts for 1 hour 45 minutes in 1XTBE running buffer.

2.6 Quantification of DNA by UV spectrophotometer analysis

About 1.5µl of DNA was diluted with distilled deionized water and read A_{230}, A_{260} and A_{280}. The A_{260} / A_{280} ratio provides an estimate of the purity of the nucleic acid. In a pure DNA sample this ratio should be around 1.8. lower values indicate protein or phenol. A_{230} should be less than A_{260} and maybe same as the A_{280}. An A_{260} of 1 corresponds to approximately 50µg/ml of double-stranded DNA in a 1cm quartz cuvette. The nucleic acid calculation are as follows;

A_{260} X 50mg / µl X 0.001µl / ml X dilution factor (1500 µl / 1.5µl)

2.7 Primers

Two sets of primers that were used for this study were taken based on published information (S.L AU *et al.*, 2001; Villand *et al.*, 1992). The sequence of these primers are shown in Table 1.

2.8 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was done by using Taq DNA polymerase and dNTP set obtained from Fermentas. The PCR mixture included template DNA molecule, two primers, 10mM dNTP mix, 10X PCR buffer without Magnesium Chloride, 25mM $MgCl_2$, *Taq* DNA Polymerase and sterile water.

PCR was carried out in the Perkin-Elmer Cetus DNA Thermal Cycler 2400. 10 trials has been done due to obtain the PCR product. Those trials are shown in Table 2.

PRIMERS	SEQUENCE
AGP-S	5'-AA(A/G)TA(T/C)CC(A/T)TA(T/C)AT(T/C/A)GCII(C/G)IATGGG-3'
AGP-AS	5'-CC(T/A)ATIC(G/T)IGC(A/G)TT(C/T)(A/T)T(A/G)TCIAT-3'
SCAGPL-S	5'-TGATGGCTACTGGGAGGACATT-3'
SCAGPL-AS	5'-ACAAGAACGGAGCCCAACCACA-3'

Table 1. Primers used for amplification of AGPase gene.

Table 2. Trials of PCR analysis

Trial 1 :

a) Master Mix PCR

COMPONENT	VOLUME (µL)
Sterile water	16.5
10X PCR Buffer without Magnesium chloride ($MgCl_2$)	2.5
Magnesium chloride ($MgCl_2$)	1
Mixtures of dNTPs	0.5
Primers	1 each
Taq polymerase	0.5
DNA template	2

b) PCR program

STEP	TEMPERATURE (°C)	TIME
First denaturing	96	5 minutes
Denaturing	95	35 seconds
Annealing	62	30 seconds
Extension	72	7 minutes
Last extension	4	Forever

Trial 2 :

a) Master Mix PCR

COMPONENT	VOLUME (μL)
Sterile water	33
10X PCR Buffer without Magnesium chloride ($MgCl_2$)	5
Magnesium chloride ($MgCl_2$)	2
Mixtures of dNTPs	1
Primers	2 each
Taq polymerase	1
DNA template	4

b) PCR program

STEP	TEMPERATURE (˚C)	TIME
First denaturing	96	5 minutes
Denaturing	95	35 seconds
Annealing	62	30 seconds
Extension	70	7 minutes
Last extension	4	Forever

Trial 3 :

a) Master Mix PCR

COMPONENT	VOLUME (μL)
Sterile water	33
10X PCR Buffer without Magnesium chloride ($MgCl_2$)	5
Magnesium chloride ($MgCl_2$)	2
Mixtures of dNTPs	1
Primers	2 each
Taq polymerase	1
DNA template	4

b) PCR program

STEP	TEMPERATURE (°C)	TIME
First denaturing	96	5 minutes
Denaturing	95	35 seconds
Annealing	52	30 seconds
Extension	72	7 minutes
Last extension	4	Forever

Trial 4 :

a) Master Mix PCR

COMPONENT	VOLUME (µL)
Sterile water	11
10X PCR Buffer without Magnesium chloride ($MgCl_2$)	4
Magnesium chloride ($MgCl_2$)	2
Mixtures of dNTPs	1
Primers	2each
Taq polymerase	1
DNA template	4

b) PCR program

STEP	TEMPERATURE (°C)	TIME
First denaturing	96	5 minutes
Denaturing	95	35 seconds
Annealing	60	30 seconds
Extension	72	7 minutes

Trial 5 :

a) Master Mix PCR

COMPONENT	VOLUME (μL)
Sterile water	13
10X PCR Buffer without Magnesium chloride ($MgCl_2$)	2.5
Magnesium chloride ($MgCl_2$)	1.5
Mixtures of dNTPs	1.0
Primers	2.0 each
Taq polymerase	1
DNA template	2

b) PCR program

STEP	TEMPERATURE (°C)	TIME
First denaturing	96	5 minutes
Denaturing	95	35 seconds
Annealing	62	30 seconds
Extension	72	7 minutes

Trial 6 :

a) Master Mix PCR

COMPONENT	VOLUME (μL)
Sterile water	9
10X PCR Buffer without Magnesium chloride ($MgCl_2$)	3
Magnesium chloride ($MgCl_2$)	2
Mixtures of dNTPs	1
Primers	3 each
Taq polymerase	2
DNA template	2

b) PCR program

STEP	TEMPERATURE (°C)	TIME
First denaturing	96	5 minutes
Denaturing	95	35 seconds
Annealing	72	30 seconds
Extension	72	7 minutes

Trial 7 :

a) Master Mix PCR

COMPONENT	VOLUME (μL)
Sterile water	13.5
10X PCR Buffer without Magnesium chloride ($MgCl_2$)	2.5
Magnesium chloride ($MgCl_2$)	1.5
Mixtures of dNTPs	0.5
Primers	2each
Taq polymerase	1
DNA template	2

b) PCR program

STEP	TEMPERATURE (°C)	TIME
First denaturing	95	5 minutes
Denaturing	94	35 seconds
Annealing	70	30 seconds
Extension	72	7 minutes

Trial 8 :

a) Master Mix PCR

COMPONENT	VOLUME (μL)
Sterile water	11
10X PCR Buffer without Magnesium chloride ($MgCl_2$)	3
Magnesium chloride ($MgCl_2$)	2.5
Mixtures of dNTPs	1.5
Primers	2 each
Taq polymerase	1
DNA template	2

b) PCR program

STEP	TEMPERATURE (˚C)	TIME
First denaturing	95	5 minutes
Denaturing	94	35 seconds
Annealing	75	30 seconds
Extension	72	7 minutes

Trial 9 :

a) Master Mix PCR

COMPONENT	VOLUME (μL)
Sterile water	11
10X PCR Buffer without Magnesium chloride ($MgCl_2$)	3
Magnesium chloride ($MgCl_2$)	2.5
Mixtures of dNTPs	1.5
Primers	2 each
Taq polymerase	1
DNA template	2

b) PCR program

STEP	TEMPERATURE (°C)	TIME
First denaturing	95	5 minutes
Denaturing	94	35 seconds
Annealing	62	30 seconds
Extension	72	7 minutes

Trial 10 :

a) Master Mix PCR

COMPONENT	VOLUME (μL)
Sterile water	11
10X PCR Buffer without Magnesium chloride ($MgCl_2$)	3
Magnesium chloride ($MgCl_2$)	2.5
Mixtures of dNTPs	1.5
Primers	2 each
Taq polymerase	1
DNA template	2

b) PCR program

STEP	TEMPERATURE (°C)	TIME
First denaturing	95	5 minutes
Denaturing	94	35 seconds
Annealing	50	30 seconds
Extension	72	7 minutes